INVESTIGATION OF HAIR FOLLICLE AND PLASMA BIOMARKERS FOR LOW-LEVEL VX VAPOR EXPOSURE.

E.M. LaFiandra, A.E. Chambers, J. Horsmon, J. J. Valdes, and J. W. Sekowski*
Molecular Engineering Team

B. Benton, M. Horsmon, R. Mioduszewski, and S. Thomson
Toxicology Team
U. S. Army Research, Development and Engineering Command, Edgewood Chemical Biological Center,
Aberdeen Proving Ground, MD 21010, USA

C. Evans and K. Lee
Virginia Bioinformatics Institute, Virginia Polytechnic University, Blacksburg, VA

A. Higgins
Icoria, Inc.
RTP, NC

1. ABSTRACT

Currently, there are no field-ready and expedient assays able to determine whether someone has been exposed to very low-level chemical warfare agent (CWA). Furthermore, if it were known that a person had been exposed, there is a paucity of information regarding the potential for transient or persistent neurological or other injuries. While our laboratory is working to address both of these problems, our recent work toward finding a more expedient method of detecting low-level agent exposure is addressed in this manuscript.

Our laboratory has used two approaches on three different tissues in order to identify molecular indicators (biomarkers) of exposure to VX, an organophosphate (OP) nerve agent. The work described in this manuscript utilizes a whole mount immunohistochemistry (IHC) technique on whisker hair follicles and liquid chromatography coupled to mass spectrometry (LC/MS) on blood plasma to mine for metabolic biomarkers of VX exposure. To date, the work has identified several promising proteins in the follicle and a panel of interesting plasma metabolite markers in the plasma and liver that can discriminate VX exposure versus air as well as level of VX exposure.

2. INTRODUCTION

In this time of heightened terrorist activity in the world, we are acutely aware of the possibility that chemical nerve agents may be used against military personnel and/or civilians. In addition to many immediate causalities, a terrorist scenario involving CWA creates a situation where many individuals are, or suspect they may have been, exposed to low, sub-symptomatic levels of agent. To date, the most common physiologically-based tests of OP exposure for either military or civilian individuals are the measurement of blood acetylcholinesterase activity or urinary metabolites. However, neither of these tests is expedient in the event of a mass exposure scenario. Thus, a rapid, reliable, dosimetric, and less-invasive test would be of tremendous value in the event of a civilian terrorist attack or an accidental low-level exposure to military personnel.

Exposure to toxic insults which do not elicit overt symptoms may still have deleterious long-term effects. There are numerous specific biochemicals, often referred to as biomarkers, that are increasingly sought after as surrogate toxicity endpoints in the field of toxicology. Once validated, biomarkers allow more rapid screening for exposure to or effects of a toxic agent.

Both blood plasma and the active hair follicle carry a large number of proteins that are responsive to toxicant exposure. They are also easily accessible for testing and therefore excellent candidates from which to mine biomarkers. Thus, our laboratory has begun work to identify protein biomarkers in follicles and metabolite biomarkers in the plasma of animals exposed to low-level VX vapor.

2.1 The Hair Follicle

On average, 90% of the skin on the human body supports some type of hair growth. Hair grows out of specialized involutions of epidermal tissue called follicles (Joseph et al, 1997). The part of the hair that is directly connected to these involutions, also called the follicle, is the growing, active part of the hair. Within the hair follicle, or bulb, comprises the largest area of metabolically active cells of the hair. These cells derive nutrients for hair growth and support from circulating blood (Adachi, 1973). Chemicals or heavy metals present

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in the body are brought into contact with the hair follicle by the circulating blood. As such, hair follicle bulb cells are responsive in real-time or near real-time to toxic insults in much the same way as skin cells. Metals, pesticides, and certain drugs may enter the hair follicle and potentially become incorporated into the hair shaft by the proliferating matrix cells found in the hair bulb (Joseph et al.). The possibility of having certain materials incorporated into the hair shaft has led to the use of this section of the hair to gauge exposure to these materials. Termed segmental hair analysis, this has become a popular method to determine exposure to drugs of abuse (e.g. cocaine, amphetamine, methamphetamine, heroin, marijuana, and steroids), therapeutic drugs (e.g. ephedrine, benzodiazepines, and barbiturates), and inorganic heavy metals (e.g. arsenic, lead, and mercury) (Villian et al., 2004; Kintz et al., 2003; Speiher et al., 2000; Nakahara et al., 1999).

2.2 Detecting Pesticides in Hair using Segmental Hair Analysis

Since OP nerve agents are chemically related to OP pesticides, we were encouraged by some recent reports suggesting that long-term exposure to pesticides (carbamate, organochlorine, and organophosphate (OP)) can be detected using segmental hair analysis. All of these studies demonstrated that pesticides (specifically, PCB, DDT, Lindane, and diazinon) could be detected in human hair (Tutudaki et al., 2005; Covaci et al., 2002; Tsakis et al., 1998). While we could not find any studies examining OP nerve agents in the hair shaft or follicle, we hypothesized that their similarity in chemical structure to OP pesticides (such as diazinon) might indicate that OP nerve agents may have an effect on the living hair follicle cells.

2.3 Development of a Novel Hair Follicle IHC Method

To overcome the risk of false positives associated with segmental hair analysis and the more invasive collection required for serum and urinalysis, we initiated work to develop a new method using intact, whole cells in the plucked hair follicle bulb. The new method described in this manuscript relies upon detection of OP agent responsive proteins (biomarkers) in intact, plucked hair follicle bulbs using a novel immunohistochemistry (IHC) method. Rather than measuring the toxicant or its metabolites, changes in specific toxicant responsive follicle bulb proteins are monitored to determine toxicant exposure. In order to begin with some known or suspected biomarkers of OP compound exposure, the OP pesticide literature was mined and 17 potential biomarkers were chosen for this work (see Table 1). This manuscript describes the identification of these potential biomarkers in the Sprague-Dawley rat whisker bulb and the modifications to the classical IHC procedure used to measure them.

2.4 Mining blood plasma for biomarkers

In order to have a complementary test for VX exposure, studies have been initiated to mine blood plasma for reliable metabolic biomarkers of VX exposure. The goal is to identify a panel of metabolites that can serve in a rapid test as an identifying biomolecular “fingerprint” of VX exposure.

Aside from the nervous system, the blood is the body’s next most valuable conduit of messages. Carried by the blood, a wide variety of molecules and molecular complexes, including hormones, enzymes, single and polypeptides and metabolites are transported through the body. In fact, plasma represents the largest repository of proteins in a single human sample. However, the majority of the plasma (about 90%) is made up of about ten proteins. Fortunately, technologies are rapidly evolving to help separate out the important signaling or “tell-tale” molecules from the vast background of protein information carried in the blood plasma. By separating out the vast protein background (i.e. albumin and IgGs), in plasma, mass spectrometry can be used to identify unique molecular signatures that arise following exposure to a nerve agent such as VX. By identifying these unique molecules in exposed animals we hope to gather information that will lead to the development rapid, reliable, and cost-efficient blood test for low-level nerve agent exposure.

3. MATERIALS AND METHODS

3.1 Collection of Rat Whiskers

Whiskers of male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were pulled manually, taking care to remove intact whisker bulbs. Samples were immediately snap frozen in liquid nitrogen and stored at –135 ^{o}C until analysis.

3.2 Antibodies for Protein Detection

A variety of commercially available primary antibodies were used in this work. Specific antibodies are listed in the figures. Secondary antibodies were fluorescently-labeled with Cy5, Cy3, or Alexa Fluor 532, or were alkaline phosphatase-conjugated.

3.3 SDS-PAGE and Western Blotting of Hair Protein

Protein was extracted from the bulb region of 15 to 25 hairs from donor rats. The bulb region was incubated in buffer as described by Inoue et al (2001).. Following extraction, the proteins were processed and separated by SDS-PAGE exactly as described in Chambers. et al. (2006). Following electrophoresis, the proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH) in a BioRad Mini Protean Transfer Cell (BioRad, Hercules, CA). Following transfer, the nitrocellulose membranes were incubated and processed exactly as described in Chambers, et al. (2006).

3.4 Immunohistochemistry (IHC)

All hair follicle IHC was carried out as described in Chambers et al. (2006). Briefly, hair samples were fixed in fresh acetone, and rinsed and incubated in TBS. The hairs were then mounted onto glass slides, leaving the bulb and lower shaft of the follicle free. Hair follicles were then digested with Proteinase K to remove the outer root sheath (ORS). Following this preparation, the lower shaft of the individual hair follicles were incubated with primary antibodies for previously identified proteins of interested followed by incubation with CY5-labeled secondary antibodies. Hair follicles were then incubated with antibodies to two growth stage-linked proteins, TNAP and MMP-2, followed by incubation with CY3- and Alexa Fluor 532-labeled secondary antibodies, respectively. This allowed for concurrent determination of expression of the protein of interest and the growth stage indicator proteins. Images were analyzed on an Affymetrix 428 scanner (Affymetrix, Santa Clara, CA) using Affymetrix Jaguar 2.0 software (Affymerix, Santa Clara, CA).

3.5 Mass Spectrometry of Plasma

Metabolomic analysis was conducted using an LC/MS platform. Results were annotated using a proprietary reference library (Icoria, Inc., RTP, NC) that allows assignment of spectral peaks to specific compounds.

3.5.1 LCMS sample preparation

Samples were prepared for analysis by extraction with aqueous acetonitrile. The extract contained internal standards that were carried through the entire sample preparation procedure. Samples were prepared in triplicate, randomized, and analyzed in a 96 well plate format.

The LCMS platform was comprised of a Bruker time of flight (TOF) instrument coupled to an HPLC. Briefly, a typical 10 µL sample was injected into an Agilent 1100 series HPLC system. LC separation was performed on an Atlantis® dC_{18}, 3 µm, 2.1 × 100 mm column (Waters, MA, USA) equilibrated with a buffer containing 5mM ammonium acetate (pH 5.5) in H_2O.

3.5.2 Data Preprocessing and Quality Control.

LC/MS peaks from each sample were aligned by mass to charge (m/z) ratio and retention time (RT) across all samples for each matrix. LC/MS components for each replicate of each sample were represented mathematically as a vector. Before analyzing the data we conducted multiple preprocessing steps, including (1) normalization to an internal standard (e.g., d3-methionine), 2) data quality assessment of technical variability, and 3) distribution of relative intensity and log transformation. Biologically, this transformation enables consideration of low concentration metabolites that capture subtle but important effects. We assessed quality of replication by comparing metabolomic profiles for each subject using hierarchical agglomerative clustering with Pearson correlation as the distance metric. Technical replicates were found to group together consistently.

3.5.3 Statistical Analysis

3.5.3.1 T-tests were conducted to identify the significantly perturbed components in the plasma by comparison against the control samples.

3.5.3.2 F-tests were conducted to identify those metabolomic components that are significantly perturbed in response to dose, time, and dose-time interaction.

3.5.3.3 Multivariate unsupervised analyses were conducted to study the global patterns of the data. An unbiased quantitative assessment of the separation between the subjects in each dose group was conducted using an unsupervised learning approach based on hierarchical agglomerative clustering of the metabolomic profiles for the subjects.

3.5.4. Component annotation

Each peak group within a biochemical profile was compared to an in-house reference library of known components. This library was created by measuring the retention time, m/z, and intensity for a series of standards using the LCMS platform. If a match cannot be found in the standard library for a particular peak group, external sources of metabolic information, such as KEGG, Brenda, PubChem, were used.

4. RESULTS

4.1 Western Blot Confirmation

All potential hair follicle targets investigated in this study were selected due to their role in skin biology or OP pesticide or nerve agent response. Western Blots were performed to ensure that these proteins of interest were located in the hair bulb. Figure 1 shows Western blotting confirmation of all targets. Of the 17 potential targets, all could be verified in the bulb region of the follicle except Na^+/K^+-ATPase α1.

Figure 1.

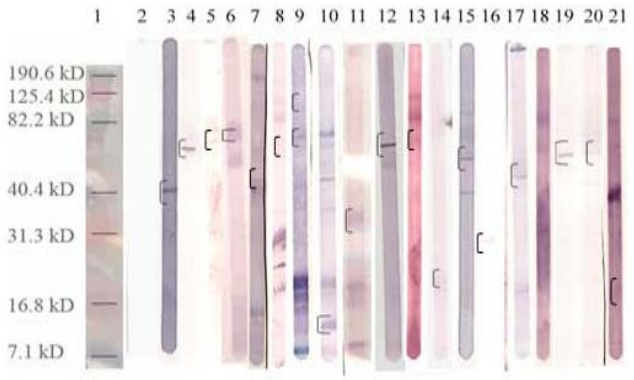

Figure 1: Western Blot of total rat follicle bulb protein separated by 10-20% SDS-PAGE. The lanes are: 1: BioRad Kaleidoscope Molecular Weight Ladder; 2: BRCA 1; 3: GAPDH; 4: Keratin 10; 5: MMP-2; 6: TNAP; 7: Actin; 8: ALDH 1A2; 9: AR; 10: BDNF; 11: CD 20; 12: CYP 1A1; 13: ER α; 14: GDNF; 15: GLUT 1; 16: GST; 17: Na^+/K^+-ATPase β1; 18: NQO I; 19: α–Tubulin; 20: β-Tubulin; 21: TNF-α

4.2 Intact Hair Follicle Bulb IHC

In order to determine whether the potential targets found by Western Blotting were also in the intact follicle, whole mount IHC preparations for the target proteins were performed (Figure 2).

The growth stage of the hair follicle greatly influences the overall activity and sensitivity of the living follicle cells. Thus, in order to compare follicles in the same growth stage, reactivity to growth stage-linked proteins, MMP-2 and TNAP, was determined for each follicle (Figure Fig. 2, photos H. and I).

In order to ensure that observed immunoreactivity results were not due to non-specific binding of the secondary antibody, negative control experiments were performed. The chicken, goat, and rabbit secondary antibodies used in the IHC all produced minimal background immunoreactivity (Figure 2B-C and 2E). However, using a mouse secondary antibody resulted in slightly higher background levels (Figure 2D). This increased background observed when using a mouse secondary antibody was likely the result of the significant protein homology between the rat and mouse. Based on these results, it was determined that polyclonal antibodies produced in chicken, goat, and rabbit should be used if possible.

An additional negative control antibody, anti-BRCA 1, was employed in these experiments. BRCA 1 proteins are not found in the hair follicle, and, as expected, no fluorescence was observed in the hair follice (fig. 2A).

Figure 2.

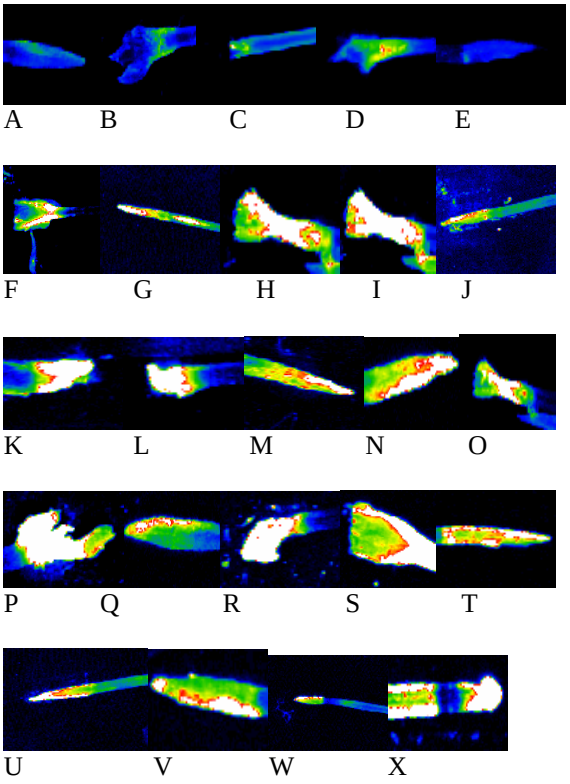

Figure 2: Fluorescence scanning images of rat whisker follicle bulbs from IHC of negative controls and confirmed targets. The figures are as follows: A: BRCA 1; B: chicken secondary negative control; C: goat secondary negative control; D: mouse secondary negative control; E: rabbit secondary negative control; F: GAPDH; G: Keratin 10; H: MMP-2; I: TNAP; J: Actin; K: ALDH 1A2; L: AR; M: BDNF; N: CD 20; O: CYP 1A1; P: ER α; Q: GDNF; R: Glut 1; s: GST; T: NQO I; U: Na^+/K^+-ATPase β1; V: α-Tubulin; W: β-Tubulin; and X: TNF-α.

4.3 Metabolites in Plasma

The metabolomic profiles of plasma and liver of 109 rats (39 air-exposed, 35 low-level (0.0004 mg/mg^3, 240 minutes) and 40 high-level (0.013 mg/m^3 , 240 minutes) were determined using liquid chromatography coupled with mass spectrometry (LC/MS) in collaboration with Icoria, Inc. Briefly, the LC/MS data was then mined for perturbed components using parametric and non-parametric statistical methods. Unsupervised analyses were also conducted using hierarchical clustering to identify groups of perturbed metabolic components in the liver and in the plasma (Table 1).

As would be expected given a typical dose-response scenario, the liver from the high dose animals exhibited a greater number and magnitude of perturbed metabolites than the liver from low dose animals. This

could be due to the greater concentration of toxicant in the liver triggering involvement of more metabolic processes in order to ameliorate the greater toxicity. The plasma appears to be a fruitful source of potentially informative markers of exposure. The metabolites with the largest degree of change in the plasma are shown in Table 2. From this pilot study we were able to glean encouraging leads that need to be verified by repetition with other samples.

Table 1. Summary of Numbers of Significantly Perturbed Metabolites By Dose and Tissue/Biofluid

Tissue	Dose	Total	P<0.05	P<0.01
Plasma	Low	975	64	12
Plasma	High	1034	70	32
Liver	Low	1763	71	13
Liver	High	1763	160	50

Table 2. Plasma Components with Largest change in response

5_hydroxyxanthotoxin	**L-Methionine**
Poly- L – Glutamate	**L-Valine**
Pyridoxal	**2-Naphthylamine**
Uridine	**4-oxoproline**
L-Citrulline	**5-Methyl-2-deoxyxytidine**
L- Tryptophan	**DNA**
L-Arginine	**Glycocholate**
Creatine	**N(pai)_Methyl_L –Histidine**
L-Serine	**Sulfate**
L-Glutamine	**Uridine**

3.4 2-D PAGE Analysis of Plasma from Low-Level VX exposed Rats

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was used to identify altered proteins in blood from VX exposed rats. Prior to separation and visualization by 2-D PAGE, blood plasma was depleted of albumin, an abundant plasma protein,. The first dimension of the 2-D PAGE separated components by isoelectric point (pI; the pH at which their net charge is neutral). The components were separated in the second dimension according to relative mass. Each sample was run in duplicate and an average image was generated for each sample for comparison purposes. To date, the data indicate that this technique is not sufficiently sensitive to detect the protein changes that were detected using LC/MS on the same samples (data not shown).

5. DISCUSSION

Carbamate, OP pesticides, and OP nerve agents exert their neuro-stimulatory and lethal effects through the inhibition of acetylcholinesterase (AChE). The ensuing increase in acetylcholine (Ach) in the synapses leads to overstimulation in portions of the sympathetic and parasympathetic nervous systems that control smooth muscle, cardiac muscle and exocrine glandular function (Munro et al, 1994). Urine and blood tests are available to determine the presence of OP compound hydrolysis products. Although conclusive, most hydrolysis product anlyses require derivatization prior to analysis (Noort 2002). Currently, measurement of AChE levels in the blood is the fastest and most commonly employed method to determine exposure to pesticides and nerve agents. Although this test is faster than the hydrolysis product tests, there are still several drawbacks to the AChE test due to the inherent nature of AChE. First, there is a 10-18% inter-individual variation and a 3 to 7% intra-individual variation in AChE levels (Maroni, 2000). Thus, without a known personal baseline, it is difficult to conclude if a person has been exposed to a nerve agent if they show inhibition levels of less than 20%. Secondly, due to the *de novo* synthesis of new AChE, this method cannot be used for retrospective determination of exposure (Noort 2002). Thus, a need exists to develop a new, simple, and rapid method to determine low-level exposure to VX and other OP compounds.

In order to provide an alternative to these more time-consuming, less field-amenable tests, work has begun to develop simple, rapid, and reliable tests of OP nerve agent exposure using blood plasma and hair follicles. Both tissues can easily be sample in the field with minimal training. It is envisioned that the first generation of the blood plasma test will be a finger-prick immuno-based assay akin to a diabetes glucose-level test. The far less invasive hair follicle-based test can also be performed quickly in the field. Additionally, hair samples can be easily preserved, stored, and shipped for repeat testing and for further confirmation.

While hair has been used as a reservoir for markers of other types of exposures, such as to heavy metals and both therapeutic drugs and drugs of abuse, very little work has employed IHC on intact hair follicle bulbs to investigate changes in the living cells of the hair. The growth state of living hair follicle cells changes considerably over the lifecycle of the hair. Follicles cycle through three stages: growth (anagen), involution (catagen) and rest (telogen) (Epstein, 1999). A number of proteins cycle along with the hair follicle growth stages, thus the growth stage of individual hairs can be assessed by the presence or absence of these specific proteins. Since hair follicle bulbs must be growth stage-matched in order to compare levels of proteins that change in response to exposures of interest, accurate

assessment of growth stage is extremely important. In this work, the growth stage assessment of a hair follicle is made by quantifying the expression of two growth stage-linked (control) proteins, alkaline phosphatase (AP) and matrix metalloproteinase-2 (MMP-2) (Handjiski et al., 1994; Johnson, et al., 1945; Yamazaki et al., 1999).

Alkaline phosphatase (EC 3.1.3.1) is a zinc metalloenzyme with a wide range of cellular functions. The tissue non-specific isoform of alkaline phosphatase (TNAP) was chosen for this work due to the fact that it is widely expressed in a number of tissues (Le Du et al., 2002; Harada et al., 2002). It is known that AP plays a role hair growth, as its expression is decreased or absent in early alopecia areata, an autoimmune response causing hair loss in patches (Handjiski et al., 1994).

Due to conflicting reports on the presence of AP in the hair follicle only during the growth stage, an additional growth control was used in the work reported in this manuscript (Handjiski et al., 1994; Johnson, et al., 1945). Matrix metalloproteinase-2 (MMP-2) has been shown to play a key role in remodeling and cell migration in the extracellular matrix (Philip et al, 2004). Yamazaki and colleagues (1999) observed growth stage dependent expression of MMP-2 mRNA in anterior dorsal skin of female Sprague-Dawley rats. MMP-2 mRNA was found to be strongly expressed in anagen hair follicles and weakly expressed in telogen follicles (Yamazaki et al., 1999). By probing all hair follicles with antibodies for both TNAP and MMP2, the growth stage of each hair follicle was determined with good confidence.

Prior to the initiation of the work describe herein, a literature search revealed that almost all published reports using IHC to study proteins in the hair follicle used sectioned, plucked hair follicles or skin biopsies. Only one manuscript described the use of unsectioned, plucked hair follicles in IHC. Horikawa and colleagues used IHC on the ORS of plucked hair follicles to study DOPA-negative melanocytes (Horikawa et al., 1996). Since the proteins of interest examined in the present study were not in the ORS, the method used in this work was slightly different from the method described by Horikowa. By digesting the ORS, the internal structure of the hair follicle bulb was easily examined without time-consuming sectioning.

The modified IHC methodology described in this manuscript was necessary to study the exposure-responsive proteins in intact, plucked rat whisker follicle bulbs. Unlike traditional segmental hair analysis in which the specific chemical or metabolites are extracted from the hair shaft and measured, the methods described in this report can be used to monitor real-time or near real-time changes in the levels of specific follicular bulb biomarkers to determine exposure to toxicants. To date, fifteen OP nerve agent-responsive target proteins have been confirmed as present in the whisker follicle using Western Blotting and IHC. Current studies presented herein suggest that this method may be useful in determining exposure to various doses of OP nerve agent. Other work in our laboratory suggests that this technique may also be useful for determining exposure to Royal Demolition Explosive (RDX). Thus, the IHC method and the potential biomarker targets described in this report may be useful in identifying or confirming exposures to a wide variety of military significant toxicants.

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